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(54) **METHODS AND MEANS FOR TREATING DNA REPEAT INSTABILITY ASSOCIATED GENETIC DISORDERS**

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(57) **ABSTRACT**

The current invention provides for methods and medicaments that apply oligonucleotide molecules complementary only to a repetitive sequence in a human gene transcript, for the manufacture of a medicament for the diagnosis, treatment or prevention of a cis-element repeat instability associated genetic disorders in humans. The invention hence provides a method of treatment for cis-element repeat instability associated genetic disorders. The invention also pertains to modified oligonucleotides which can be applied in method of the invention to prevent the accumulation and/or translation of repeat expanded transcripts in cells.

25 Claims, 5 Drawing Sheets

Specification includes a Sequence Listing.

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U.S. Appl. No. 14/809,483 2016/0053254 U.S. Pat. No. 9,890,379, filed Jul. 27, 2015 Feb. 25, 2016 Feb. 13, 2018, Josephus Johannes De Kimpe.
U.S. Appl. No. 15/855,848 2018/0112214 U.S. Pat. No. 10,689,646, filed Dec. 27, 2017 Apr. 26, 2018 Jun. 23, 2020, Josephus Johannes De Kimpe.

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Fig 1

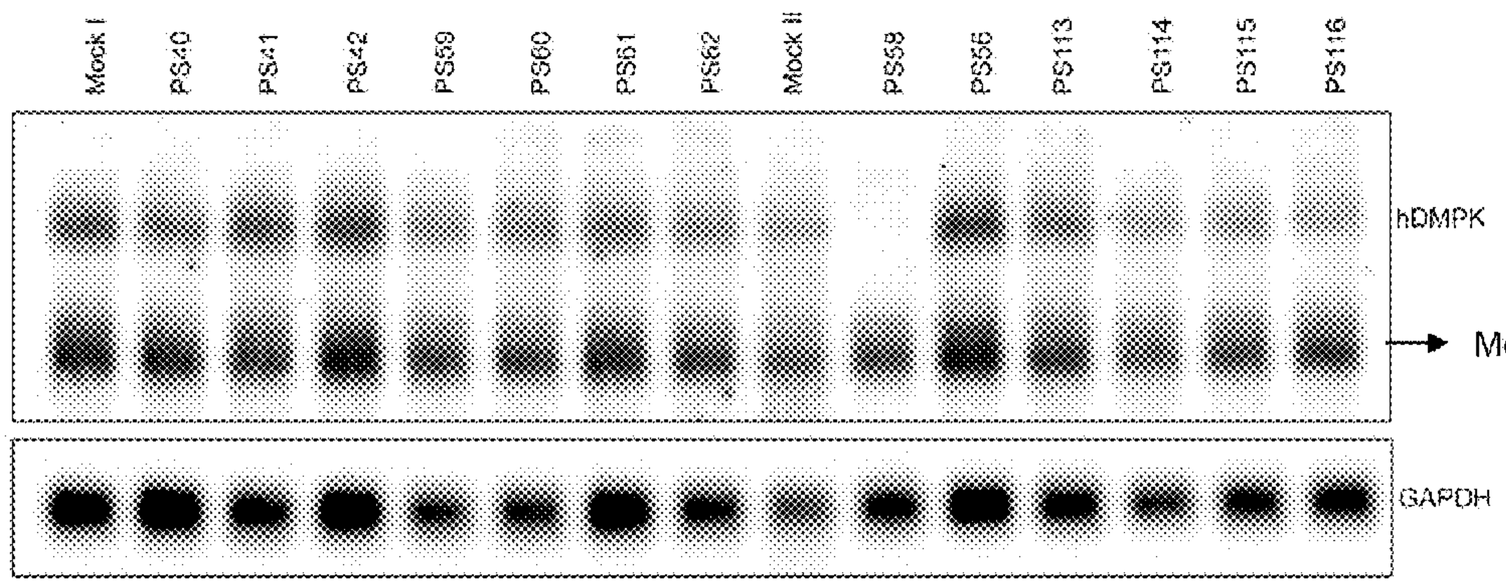

Mock I
PS40
PS41
PS42
PS59
PS60
PS61
PS62
Mock II
PS58
PS56
PS113
PS114
PS115
PS116
hDMPK
Mouse
GAPDH

Fig 2

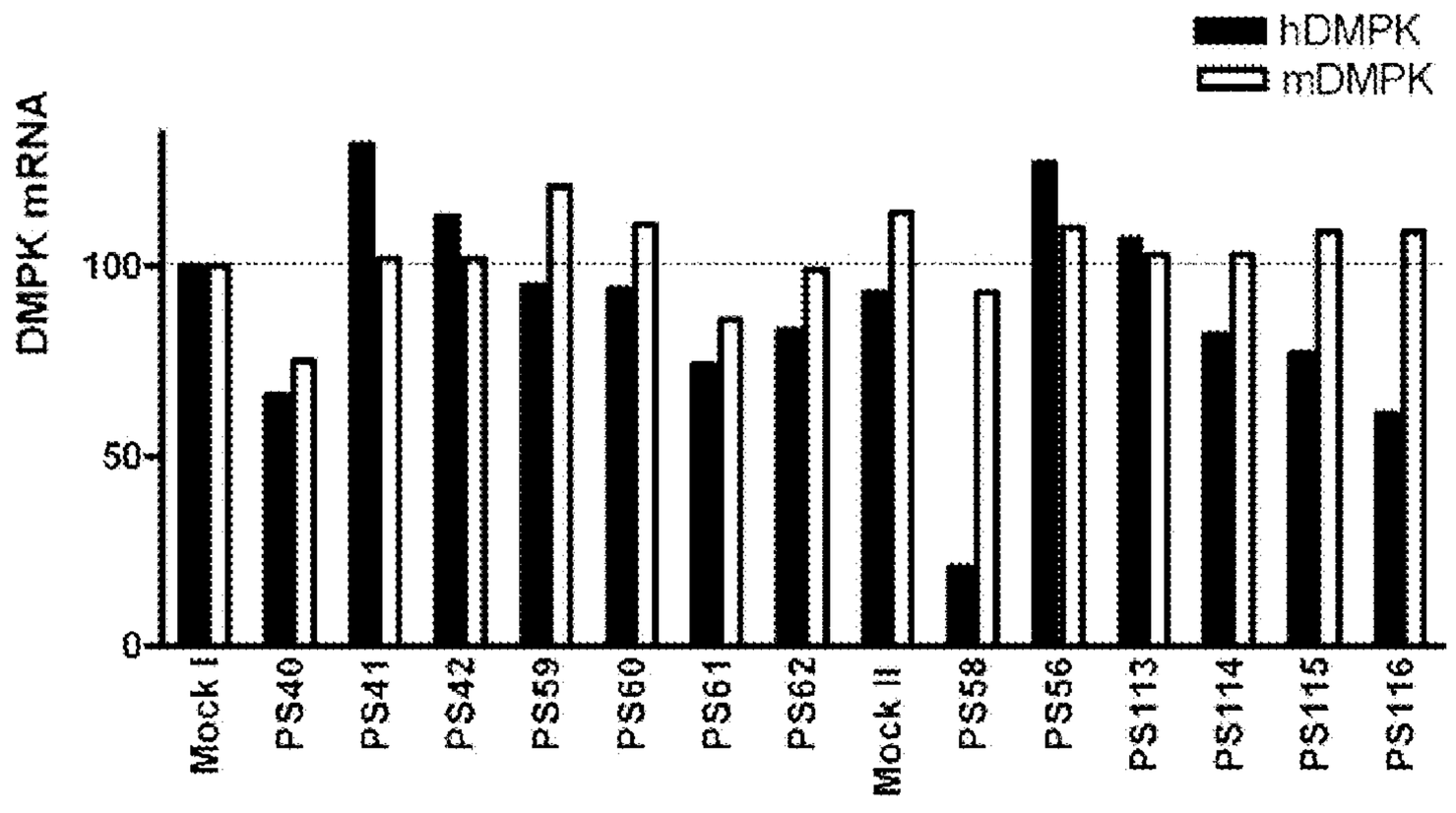

hDMPK
mDMPK
DMPK mRNA
100
50
0
Mock I
PS40
PS41
PS42
PS59
PS60
PS61
PS62
Mock II
PS58
PS56
PS113
PS114
PS115
PS116

Fig 3

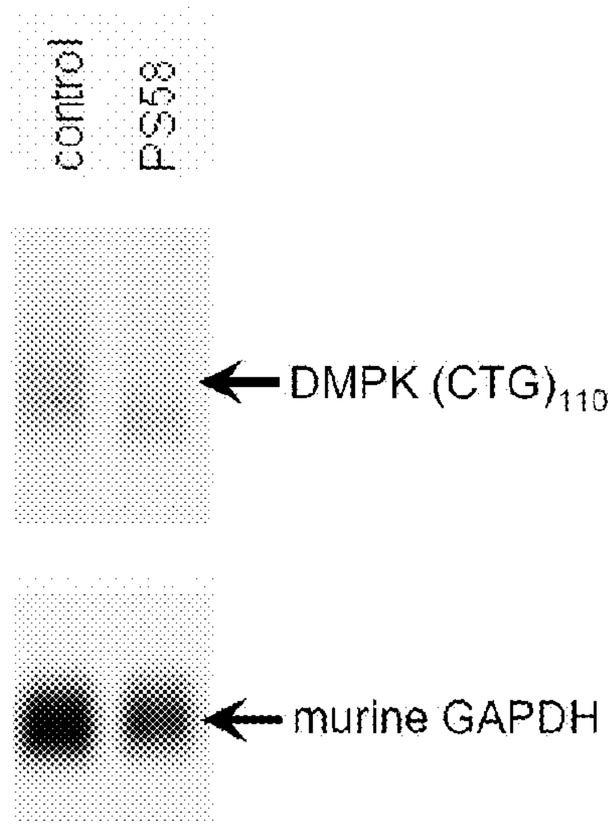

control
PS58
DMPK (CTG)110
murine GAPDH

Fig 4

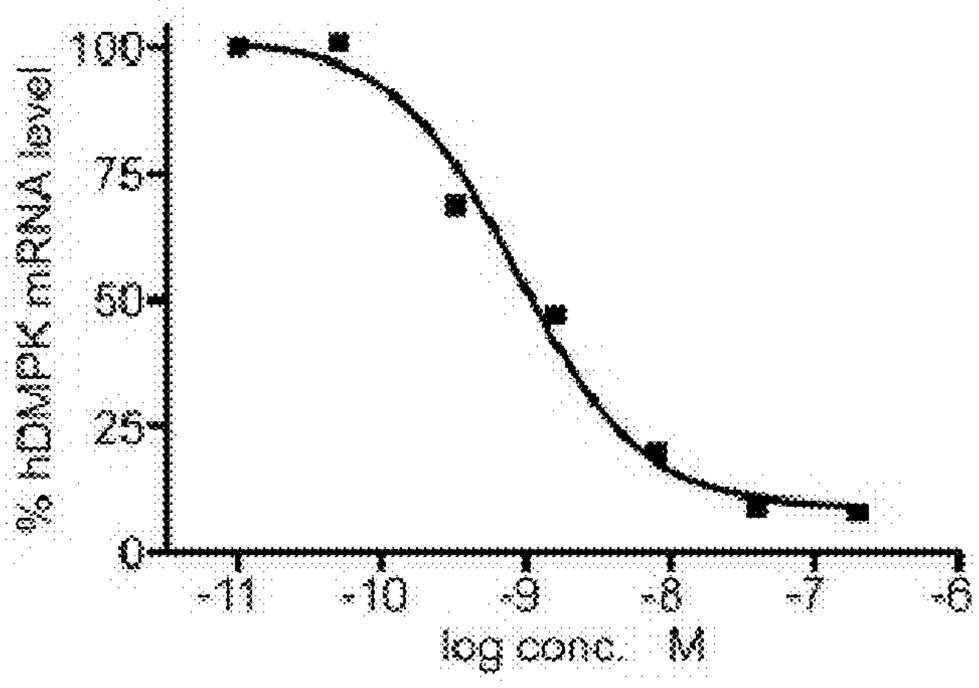

% hDMPK mRNA level
100
75
50
25
0
-11
-10
-9
-8
-7
-6
log conc. M

Fig 5

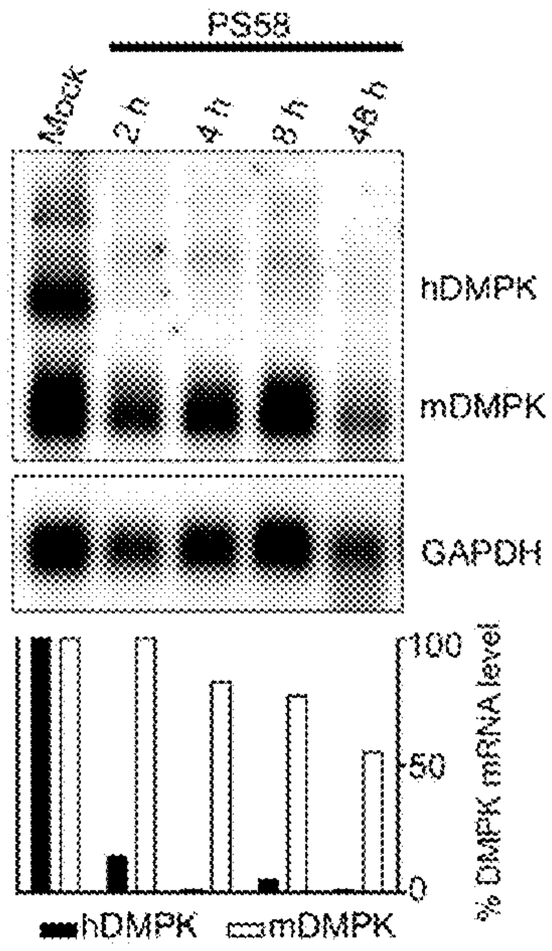


Fig 6

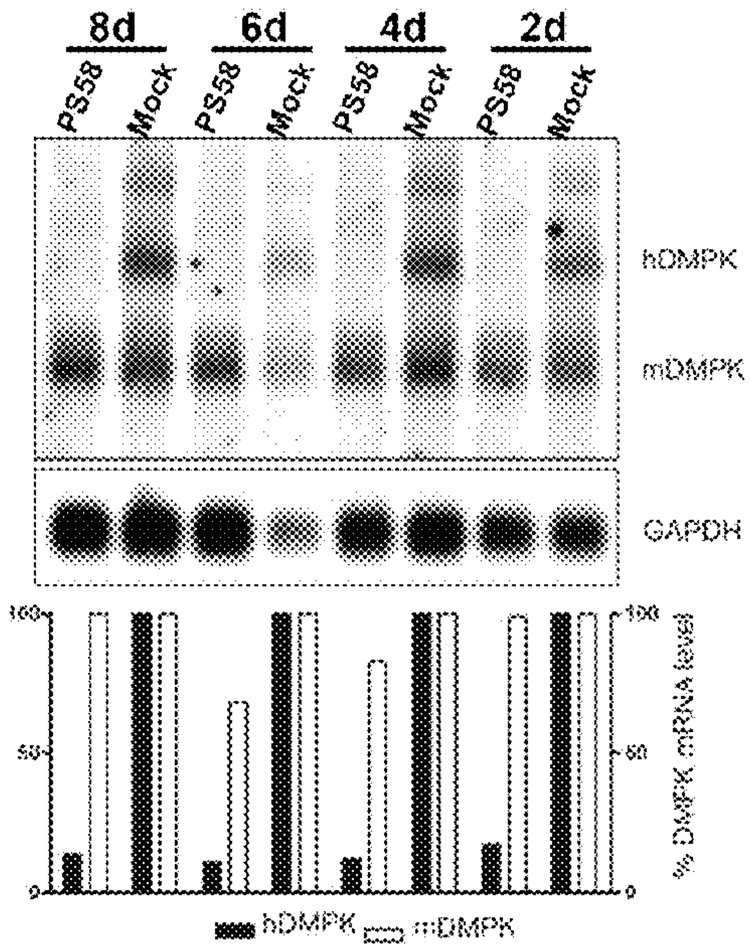


Fig 7

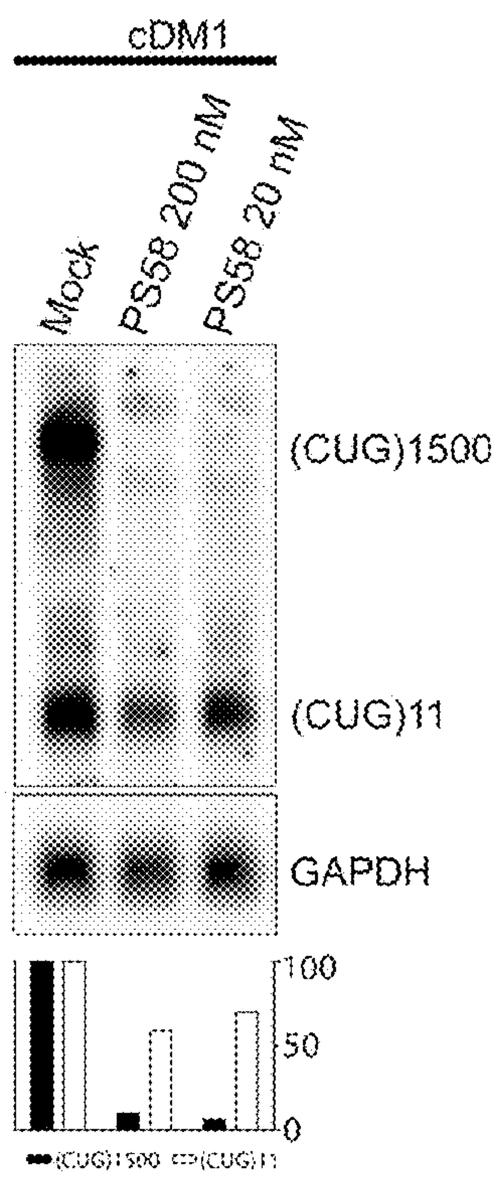


Fig 8

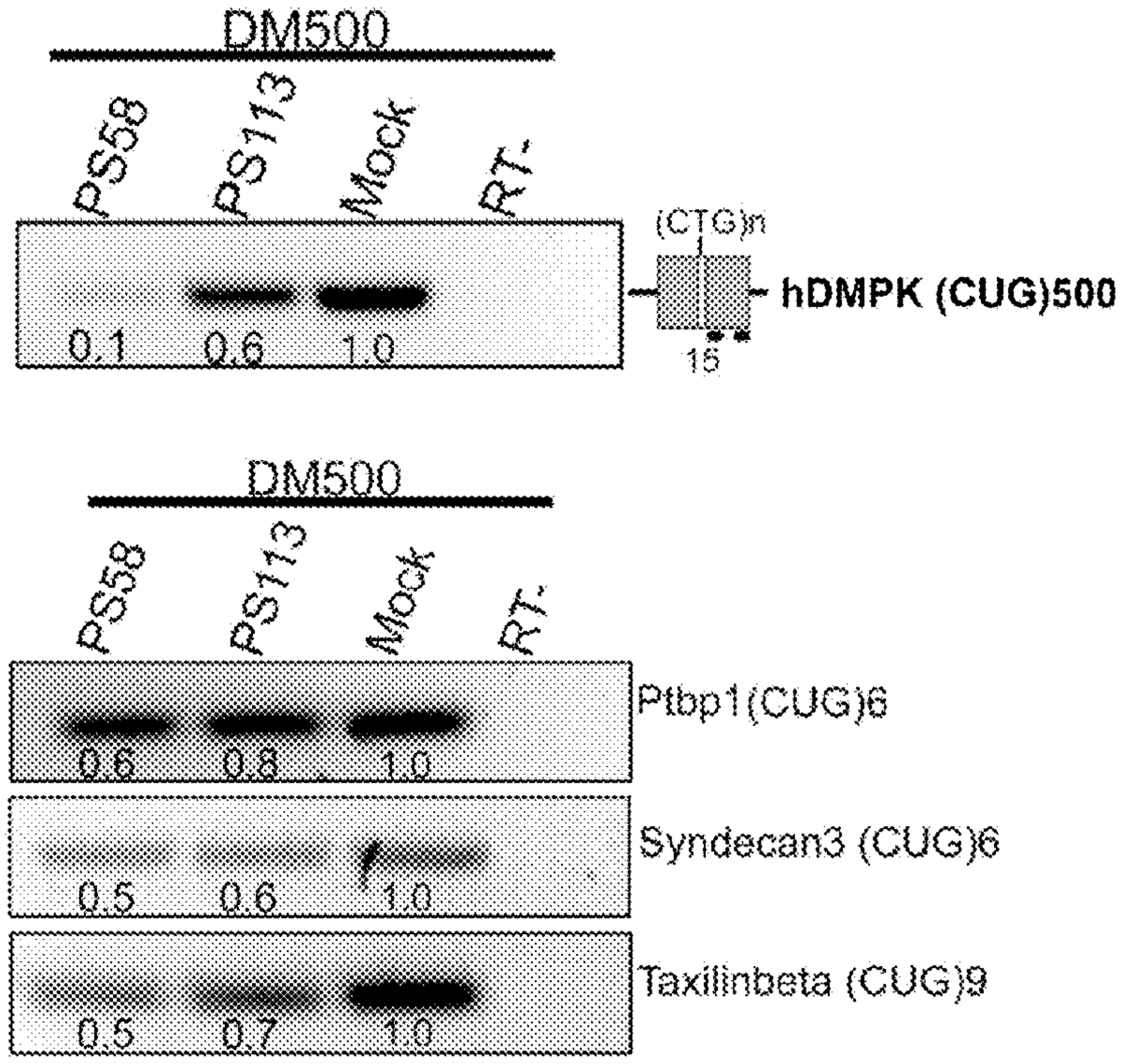


Fig 9

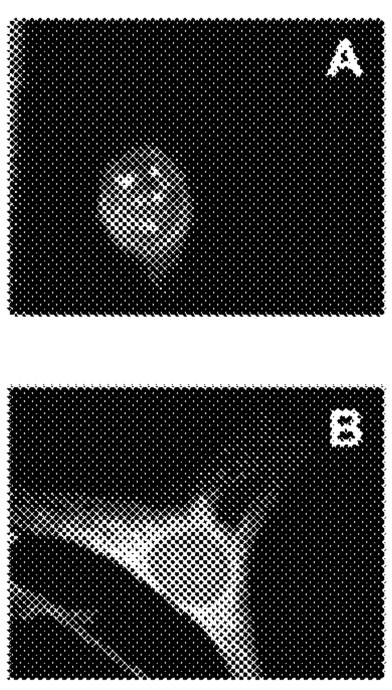


Fig 10

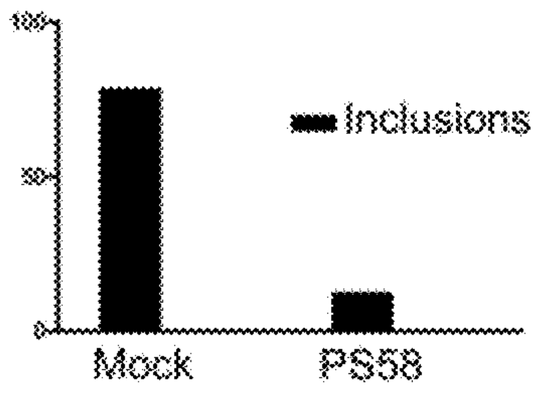


Fig 11

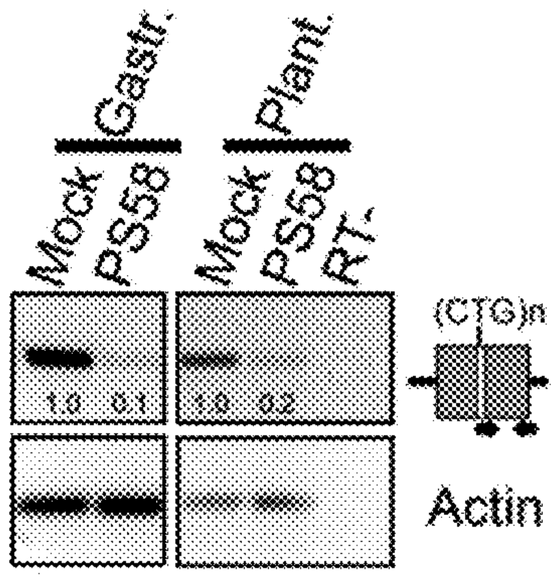
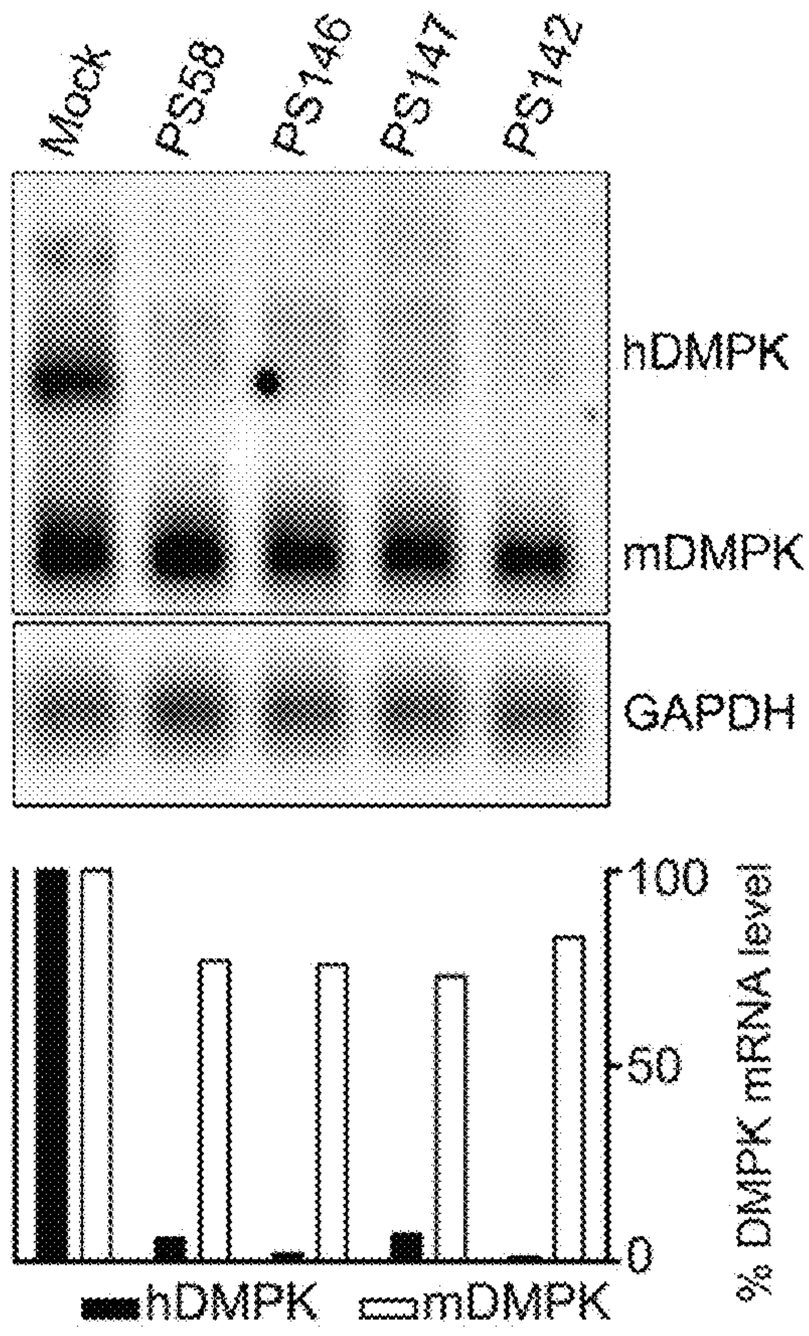

Gastr.
Plant.
Mock
PS58
Mock
PS58
RT-
(CTG)n
1.0
0.1
1.0
0.2
Actin

Fig 12


Mock
PS58
PS146
PS147
PS142
hDMPK
mDMPK
GAPDH
100
50
0
% DMPK mRNA level
hDMPK
mDMPK

METHODS AND MEANS FOR TREATING DNA REPEAT INSTABILITY ASSOCIATED GENETIC DISORDERS

RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 15/855,848, filed Dec. 27, 2017, which is a division of U.S. patent application Ser. No. 14/809,483, filed Jul. 27, 2015, now U.S. Pat. No. 9,890,379, which is a division of U.S. patent application Ser. No. 12/377,160, filed Feb. 24, 2010, which is a 35 U.S.C. § 371 filing of International Patent Application No. PCT/NL2007/050399, filed Aug. 10, 2007, which claims priority to European Patent Application Nos. 06118809.0, filed Aug. 11, 2006, and 06119247.2, filed Aug. 21, 2006, the disclosures of which are incorporated herein by reference in their entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Oct. 22, 2019, is named 617852_VCO9-001USDIV2CON_Sequence_Listing.txt and is 7,568 bytes in size.

FIELD OF THE INVENTION

The current invention relates to the field of medicine, in particular to the treatment of genetic disorders associated with genes that have unstable repeats in their coding or non-coding sequences, most in particular unstable repeats in the human Huntington disease causing HD gene or the myotonic dystrophy type 1 causing DMPK gene.

BACKGROUND OF THE INVENTION

Instability of gene-specific microsatellite and minisatellite repetitive sequences, leading to increase in length of the repetitive sequences in the satellite, is associated with about 35 human genetic disorders. Instability of trinucleotide repeats is for instance found in genes causing X-linked spinal and bulbar muscular atrophy (SBMA), myotonic dystrophy type 1 (DM1), fragile X syndrome (FRAX genes A, E, F), Huntington's disease (HD) and several spinocerebellar ataxias (SCA gene family).

Unstable repeats are found in coding regions of genes, such as the Huntington's disease gene, whereby the phenotype of the disorder is brought about by alteration of protein function and/or protein folding. Unstable repeat units are also found in untranslated regions, such as in myotonic dystrophy type 1 (DM1) in the 3' UTR or in intronic sequences such as in myotonic dystrophy type 2 (DM2). The normal number of repeats is around 5 to 37 for DMPK, but increases to premutation and full disease state two to ten fold or more, to 50, 100 and sometimes 1000 or more repeat units. For DM2/ZNF9 increases to 10,000 or more repeats have been reported. (Cleary and Pearson, Cytogenet. Genome Res. 100: 25-55, 2003).

The causative gene for Huntington's disease, HD, is located on chromosome 4. Huntington's disease is inherited in an autosomal dominant fashion. When the gene has more than 35 CAG trinucleotide repeats coding for a polyglutamine stretch, the number of repeats can expand in successive generations. Because of the progressive increase in length of the repeats, the disease tends to increase in severity and

presents at an earlier age in successive generations, a process called anticipation. The product of the HD gene is the 348 kDa cytoplasmic protein huntingtin. Huntingtin has a characteristic sequence of fewer than 40 glutamine amino acid residues in the normal form; the mutated huntingtin causing the disease has more than 40 residues. The continuous expression of mutant huntingtin molecules in neuronal cells results in the formation of large protein deposits which eventually give rise to cell death, especially in the frontal lobes and the basal ganglia (mainly in the caudate nucleus). The severity of the disease is generally proportional to the number of extra residues.

DM1 is the most common muscular dystrophy in adults and is an inherited, progressive, degenerative, multisystemic disorder of predominantly skeletal muscle, heart and brain. DM1 is caused by expansion of an unstable trinucleotide (CTG)n repeat in the 3' untranslated region of the DMPK gene (myotonic dystrophy protein kinase) on human chromosome 19q (Brook et al, Cell, 1992). Type 2 myotonic dystrophy (DM2) is caused by a CCTG expansion in intron 1 of the ZNF9 gene, (Liquori et al, Science 2001). In the case of myotonic dystrophy type 1, the nuclear-cytoplasmic export of DMPK transcripts is blocked by the increased length of the repeats, which form hairpin-like secondary structures that accumulate in nuclear foci. DMPK transcripts bearing a long (CUG)n tract can form hairpin-like structures that bind proteins of the muscleblind family and subsequently aggregate in ribonuclear foci in the nucleus. These nuclear inclusions are thought to sequester muscleblind proteins, and potentially other factors, which then become limiting to the cell. In DM2, accumulation of ZNF9 RNA carrying the (CCUG)n expanded repeat form similar foci. Since muscleblind proteins are splicing factors, their depletion results in a dramatic rearrangement in splicing of other transcripts. Transcripts of many genes consequently become aberrantly spliced, for instance by inclusion of fetal exons, or exclusion of exons, resulting in non-functional proteins and impaired cell function.

The observations and new insights above have led to the understanding that unstable repeat diseases, such as myotonic dystrophy type 1, Huntington's disease and others can be treated by removing, either fully or at least in part, the aberrant transcript that causes the disease. For DM1, the aberrant transcript that accumulates in the nucleus could be down regulated or fully removed. Even relatively small reductions of the aberrant transcript could release substantial and possibly sufficient amounts of sequestered cellular factors and thereby help to restore normal RNA processing and cellular metabolism for DM (Kanadia et al., PNAS 2006). In the case of HD, a reduction in the accumulation of huntingtin protein deposits in the cells of an HD patient can ameliorate the symptoms of the disease.

A few attempts have been made to design methods of treatment and medicaments for unstable repeat disease myotonic dystrophy type 1 using antisense nucleic acids, RNA interference or ribozymes. (i) Langlois et al. (Molecular Therapy, Vol. 7 No. 5, 2003) designed a ribozyme capable of cleaving DMPK mRNA. The hammerhead ribozyme is provided with a stretch RNA complementary to the 3' UTR of DMPK just before the CUG repeat. In vivo, vector transcribed ribozyme was capable of cleaving and diminishing in transfected cells both the expanded CUG repeat containing mRNA as well as the normal mRNA species with 63 and 50% respectively. Hence, also the normal transcript is gravely affected by this approach and the affected mRNA species with expanded repeats are not specifically targeted.

(ii) Another approach was taken by Langlois et al., (Journal Biological Chemistry, vol 280, no. 17, 2005) using RNA interference. A lentivirus-delivered short-hairpin RNA (shRNA) was introduced in DM1 myoblasts and demonstrated to down regulate nuclear retained mutant DMPK mRNAs. Four shRNA molecules were tested, two were complementary against coding regions of DMPK, one against a unique sequence in the 3' UTR and one negative control with an irrelevant sequence. The first two shRNAs were capable of down regulating the mutant DMPK transcript with the amplified repeat to about 50%, but even more effective in down regulating the cytoplasmic wildtype transcript to about 30% or less. Equivalent synthetic siRNA delivered by cationic lipids was ineffective. The shRNA directed at the 3' UTR sequence proved to be ineffective for both transcripts. Hence, also this approach is not targeted selectively to the expanded repeat mRNA species.

(iii) A third approach by Furling et al. (Gene Therapy, Vol. 10, p 795-802, 2003) used a recombinant retrovirus expressing a 149-bp long antisense RNA to inhibit DMPK mRNA levels in human DM1 myoblasts. A retrovirus was designed to provide DM1 cells with the 149 bp long antisense RNA complementary to a 39 bp-long (CUG)13 repeat and a 110 bp region following the repeat to increase specificity. This method yielded a decrease in mutated (repeat expanded) DMPK transcript of 80%, compared to a 50% reduction in the wild type DMPK transcript and restoration of differentiation and functional characteristics in infected DM1 myoblasts. Hence, also this approach is not targeted selectively to the expanded repeat mRNA species, it depends on a very long antisense RNA and can only be used in combination with recombinant viral delivery techniques.

DETAILED DESCRIPTION OF THE INVENTION

The methods and techniques described above provide nucleic acid based methods that cause non-selective breakdown of both the affected repeat expanded allele and unaffected (normal) allele for genetic diseases that are associated with repeat instability and/or expansion. Moreover, the art employs sequences specific for the gene associated with the disease and does not provide a method that is applicable to several genetic disorders associated with repeat expansion. Finally, the art only teaches methods that involve use of recombinant DNA vector delivery systems, which need to be adapted for each oligonucleotide and target cell and which still need to be further optimised.

The current invention provides a solution for these problems by using a short single stranded nucleic acid molecule that comprises or consists of a sequence, which is complementary to the expanded repeat region only, i.e. it does not rely on hybridisation to unique sequences in exons or introns of the repeat containing gene. Furthermore, it is not essential that the employed nucleic acid (oligonucleotide) reduces transcripts by the RNAse H mediated breakdown mechanism.

Without wishing to be bound by theory, the current invention may cause a decrease in transcript levels by alterations in posttranscriptional processing and/or splicing of the premature RNA. A decrease in transcript levels via alternative splicing and/or postranscriptional processing is thought to result in transcripts lacking the overly expanded or instable (tri)nucleotide repeat, but still possessing functional activities. The reduction of aberrant transcripts by altered RNA processing and/or splicing may prevent accumulation and/or translation of aberrant, repeat expanded transcripts in cells.

Without wishing to be bound by theory the method of the current invention is also thought to provide specificity for the affected transcript with the expanded repeat because the kinetics for hybridisation to the expanded repeat are more favourable. The likelihood that a repeat specific complementary nucleic acid oligonucleotide molecule will hybridise to a complementary stretch in an RNA or DNA molecule increases with the size of the repetitive stretch. RNA molecules and in particular RNA molecules comprising repetitive sequences are normally internally paired, forming a secondary structure comprising open loops and closed hairpin parts. Only the open parts are relatively accessible for complementary nucleic acids. The short repeat stretches of a wild type transcript not associated with disease is often only 5 to about 20-40 repeats and due to the secondary structure relatively inaccessible for base pairing with a complementary nucleic acid. In contrast, the repeat units of the expanded repeat and disease associated allele is normally at least 2 fold expanded but usually even more, 3, 5, 10 fold, up to 100 or even more than 1000 fold expansion for some unstable repeat disorders. This expansion increases the likelihood that part of the repeat is, at least temporarily, in an open loop structure and thereby more accessible to base pairing with a complementary nucleic acid molecule, relative to the wild type allele. So despite the fact that the oligonucleotide is complementary to a repeat sequence present in both wildtype and repeat-expanded transcripts and could theoretically hybridise to both transcripts, the current invention teaches that oligonucleotides complementary to the repetitive tracts preferably hybridise to the disease-associated or disease-causing transcripts and leave the function of normal transcripts relatively unaffected. This selectivity is beneficial for treating diseases associated with repeat instability irrespective of the mechanism of reduction of the aberrant transcript.

The invention thus provides a method for the treatment of unstable cis-element DNA repeat associated genetic disorders, by providing nucleic acid molecules that are complementary to and/or capable of hybridising to the repetitive sequences only. This method thereby preferentially targets the expanded repeat transcripts and leaves the transcripts of the normal, wild type allele relatively unaffected. This is advantageous since the normal allele can thereby provide for the normal function of the gene, which is at least desirable and, depending on the particular gene with unstable DNA repeats, may in many cases be essential for the cell and/or individual to be treated.

Furthermore, this approach is not limited to a particular unstable DNA repeat associated genetic disorder, but may be applied to any of the known unstable DNA repeat diseases, such as, but not limited to: coding regions repeat diseases having a polyglutamine (CAG) repeat: Huntington's disease, Haw River syndrome, Kennedy's disease/spinobulbar muscular atrophy, spino-cerebellar ataxia, or diseases having polyalanine (GCG) repeats such as: infantile spasm syndrome, deidocranial dysplasia, blepharophimosis/ptosis/epicanthus invensus syndrome, hand-foot-genital syndrome, synpolydactyly, oculopharyngeal muscular dystrophy, holoprosencephaly. Diseases with repeats in non-coding regions of genes to be treated according to the invention comprise the trinucleotide repeat disorders (mostly CTG and/or CAG and/or CCTG repeats): myotonic dystrophy type 1, myotonic dystrophy type 2, Friedreich's ataxia (mainly GAA), spino-cerebellar ataxia, autism. Furthermore, the method of

the invention can be applied to fragile site associated repeat disorder comprising various fragile X-syndromes, Jacobsen syndrome and other unstable repetitive element disorders such as myoclonus epilepsy, facioscapulohumeral dystrophy and certain forms of diabetes mellitus type 2.

Another advantage of the current invention is that the oligonucleotides specific for a repeat region may be administered directly to cells and it does not rely on vector-based delivery systems. The techniques described in the prior art, for instance those mentioned above for treatment of DM1 and removal of DMPK transcripts from cells, require the use of vector based delivery systems to administer sufficient levels of oligonucleotides to the cell. The use of plasmid or viral vectors is yet less desirable for therapeutic purposes because of current strict safety regulations for therapeutic recombinant DNA vectors, the production of sufficient recombinant vectors for broad clinical application and the limited control and reversibility of an exaggerated (or non-specific) response after application. Nevertheless, optimisation in future is likely in these areas and viral delivery of plasmids could yield an advantageous long lasting effect. The current inventors have surprisingly found that oligonucleotides that comprise or consist of a sequence that is complementary to repetitive sequences of expanded repeat transcripts, due to the expansion of their molecular target for hybridisation, have a much increased affinity and/or avidity for their target in comparison to oligonucleotides that are specific for unique sequences in a transcript. Because of this high affinity and avidity for the repeat expanded target transcript, lower amounts of oligonucleotide suffice to yield sufficient inhibition and/or reduction of the repeat expanded allele by RNase H degradation, RNA interference degradation or altered post-transcriptional processing (comprising but not limited to splicing or exon skipping) activities. The oligonucleotides of the current invention which are complementary to repetitive sequences only, may be produced synthetically and are potent enough to be effective when delivered directly to cells using commonly applied techniques for direct delivery of oligonucleotides to cells and/or tissues. Recombinant vector delivery systems may, when desired, be circumvented by using the method and the oligonucleotide molecules of the current invention.

In a first aspect, the current invention discloses and teaches the use of an oligonucleotide comprising or consisting of a sequence that is complementary only to a repetitive sequence in a human gene transcript for the manufacture of a medicament for the diagnosis, treatment or prevention of a cis-element repeat instability associated genetic disorders in humans. The invention hence provides a method of treatment for cis-element repeat instability associated genetic disorders.

In a second aspect, the invention also pertains to an oligonucleotide which can be used in the first aspect of the invention and/or applied in method of the invention to prevent the accumulation and/or translation of repeat expanded transcripts in cells.

An oligonucleotide of the invention may comprise a sequence that is complementary only to a repetitive sequence as defined below. Preferably, the repetitive sequence is at least 50% of the length of the oligonucleotide of the invention, more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90% or more. In a most preferred embodiment, the oligonucleotide of the invention consists of a sequence that is complementary only to a repetitive sequence as defined below. For example, an oligonucleotide may comprise a sequence that is complementary only to a repetitive sequence as defined below and a targeting part, which is later on called a targeting ligand.

A repeat or repetitive element or repetitive sequence or repetitive stretch is herein defined as a repetition of at least 3, 4, 5, 10, 100, 1000 or more, of a repetitive unit or repetitive nucleotide unit or repeat nucleotide unit comprising a trinucleotide repetitive unit, or alternatively a 4, 5 or 6 nucleotide repetitive unit, in a transcribed gene sequence in the genome of a subject, including a human subject.

An oligonucleotide may be single stranded or double stranded. Double stranded means that the oligonucleotide is an heterodimer made of two complementary strands, such as in a siRNA. In a preferred embodiment, an oligonucleotide is single stranded. A single stranded oligonucleotide has several advantages compared to a double stranded siRNA oligonucleotide: (i) its synthesis is expected to be easier than two complementary siRNA strands; (ii) there is a wider range of chemical modifications possible to optimise more effective uptake in cells, a better (physiological) stability and to decrease potential generic adverse effects; and (iii) siRNAs have a higher potential for non-specific effects and exaggerated pharmacology (e.g. less control possible of effectiveness and selectivity by treatment schedule or dose) and (iv) siRNAs are less likely to act in the nucleus and cannot be directed against introns. Therefore, in a preferred embodiment of the first aspect, the invention relates to the use of a single stranded oligonucleotide comprising or consisting of a sequence that is complementary only to a repetitive sequence in a human gene transcript for the manufacture of a medicament for the diagnosis, treatment or prevention of a cis-element repeat instability associated genetic disorders in humans.

The oligonucleotide(s) preferably comprise at least 10 to about 50 consecutive nucleotides complementary to a repetitive element, more preferably 12 to 45 nucleotides, even more preferably 12 to 30, and most preferably 12 to 25 nucleotides complementary to a repetitive stretch, preferably having a trinucleotide repeat unit or a tetranucleotide repeat unit. The oligonucleotide may be complementary to and/or capable of hybridizing to a repetitive stretch in a coding region of a transcript, preferably a polyglutamine (CAG) or a polyalanine (GCG) coding tract. The oligonucleotide may also be complementary to and/or capable of hybridizing to a non-coding region for instance 5' or 3' untranslated regions, or intronic sequences present in precursor RNA molecules.

In a preferred embodiment the oligonucleotide to be used in the method of the invention comprises or consists of a sequence that is complementary to a repetitive element having as repetitive nucleotide unit a repetitive nucleotide unit selected from the group consisting of (CAG)n, (GCG)n, (CUG)n, (CGG)n (GAA)n, (GCC)n and (CCUG)n. and said oligonucleotide being a single or double stranded oligonucleotide. Preferably, the oligonucleotide is double stranded.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a polyglutamine (CAG)n tract in a transcript is particularly useful for the diagnosis, treatment and/or prevention of the human disorders Huntington's disease, several forms of spino-cerebellar ataxia or Haw River syndrome, X-linked spinal and bulbar muscular atrophy and/or dentatorubral-pallidoluysian atrophy caused by repeat expansions in the HD, HDL2/JPH3, SBMA/AR, SCA1/ATX1, SCA2/ATX2, SCA3/ATX3, SCA6/CACNAIA, SCA7, SCA17, AR or DRPLA human genes.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a polyalanine (GCG)n tract in a transcript is particularly useful for the diagnosis, treatment and/or prevention of the human disorders: infantile spasm syndrome, deidocranial dysplasia, blepharophimosis, hand-foot-genital disease, synpolydactyly, oculopharyngeal muscular dystrophy and/or holoprosencephaly, which are caused by repeat expansions in the ARX, CBFA1, FOXL2, HOXA13, HOXD13, OPDM/PABP2, TCFBR1 or ZIC2 human genes.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (CUG)n repeat in a transcript and is particularly useful for the diagnosis, treatment and/or prevention of the human genetic disorder myotonic dystrophy type 1, spino-cerebrellar ataxia 8 and/or Huntington's disease-like 2 caused by repeat expansions in the DM1/DMPK, SCA8 or JPH3 genes respectively. Preferably, these genes are from human origin.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (CCUG)n repeat in a transcript is particularly useful for the diagnosis, treatment and/or prevention of the human genetic disorder myotonic dystrophy type 2, caused by repeat expansions in the DM2/ZNF9 gene.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (CGG)n repeat in a transcript is particularly useful for the diagnosis, treatment and/or prevention of human fragile X syndromes, caused by repeat expansion in the FRAXA/FMR1, FRAXE/FMR2 and FRAXF/FAM11A genes.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (CCG)n repeat in a transcript is particularly useful for the diagnosis, treatment and/or prevention of the human genetic disorder Jacobsen syndrome, caused by repeat expansion in the FRA11B/CBL2 gene.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (GAA)n repeat in a transcript is particularly useful for the diagnosis, treatment and/or prevention of the human genetic disorder Friedreich's ataxia.

The use of an oligonucleotide that comprises or consists of a sequence that is complementary to a (ATTCT)n repeat in an intron is particularly useful for the diagnosis, treatment and/or prevention of the human genetic disorder Spinocerebellar ataxia type 10 (SCA10).

The repeat-complementary oligonucleotide to be used in the method of the invention may comprise or consist of RNA, DNA, Locked nucleic acid (LNA), peptide nucleic acid (PNA), morpholino phosphorodiamidates (PMO), ethylene bridged nucleic acid (ENA) or mixtures/hybrids thereof that comprise combinations of naturally occurring DNA and RNA nucleotides and synthetic, modified nucleotides. In such oligonucleotides, the phosphodiester backbone chemistry may further be replaced by other modifications, such as phosphorothioates or methylphosphonates. Other oligonucleotide modifications exist and new ones are likely to be developed and used in practice. However, all such oligonucleotides have the character of an oligomer with the ability of sequence specific binding to RNA. Therefore in a preferred embodiment, the oligonucleotide comprises or consists of RNA nucleotides, DNA nucleotides, locked nucleic acid (LNA) nucleotides, peptide nucleic acid (PNA) nucleotides, morpholino phosphorodiamidates, ethylene-bridged nucleic acid (ENA) nucleotides or mixtures thereof with or without phosphorothioate containing backbones.

Oligonucleotides containing at least in part naturally occurring DNA nucleotides are useful for inducing degradation of DNA-RNA hybrid molecules in the cell by RNase H activity (EC.3.1.26.4).

Naturally occurring RNA ribonucleotides or RNA-like synthetic ribonucleotides comprising oligonucleotides may be applied in the method of the invention to form double stranded RNA-RNA hybrids that act as enzyme-dependent antisense through the RNA interference or silencing (RNAi/siRNA) pathways, involving target RNA recognition through sense-antisense strand pairing followed by target RNA degradation by the RNA-induced silencing complex (RISC).

Alternatively or in addition, steric blocking antisense oligonucleotides (RNase-H independent antisense) interfere with gene expression or other precursor RNA or messenger RNA-dependent cellular processes, in particular but not limited to RNA splicing and exon skipping, by binding to a target sequence of RNA transcript and getting in the way of processes such as translation or blocking of splice donor or splice acceptor sites. Alteration of splicing and exon skipping techniques using modified antisense oligonucleotides are well documented, known to the skilled artisan and may for instance be found in U.S. Pat. No. 6,210,892, WO9426887, WO04/083446 and WO02/24906.

Moreover, steric hindrance may inhibit the binding of proteins, nuclear factors and others and thereby contribute to the decrease in nuclear accumulation or ribonuclear foci in diseases like DM1.

The oligonucleotides of the invention, which may comprise synthetic or modified nucleotides, complementary to (expanded) repetitive sequences are useful for the method of the invention for reducing or inactivating repeat containing transcripts via the siRNA/RNA interference or silencing pathway.

Single or double stranded oligonucleotides to be used in the method of the invention may comprise or consist of DNA nucleotides, RNA nucleotides, 2'-0 substituted ribonucleotides, including alkyl and methoxy ethyl substitutions, peptide nucleic acid (PNA), locked nucleic acid (LNA) and morpholino (PMO) antisense oligonucleotides and ethylene-bridged nucleotides (ENA) and combinations thereof, optionally chimeras with RNAse H dependent antisense. Integration of locked nucleic acids in the oligonucleotide changes the conformation of the helix after base pairing and increases the stability of the duplex. Integration of LNA bases into the oligonucleotide sequence can therefore be used to increase the ability of complementary oligonucleotides of the invention to be active in vitro and in vivo to increase RNA degradation inhibit accumulation of transcripts or increase exon skipping capabilities. Peptide nucleic acids (PNAs), an artificial DNA/RNA analog, in which the backbone is a pseudopeptide rather than a sugar, have the ability to form extremely stable complexes with complementary DNA oligomers, by increased binding and a higher melting temperature. Also PNAs are superior reagents in antisense and exon skipping applications of the invention. Most preferably, the oligonucleotides to be used in the method of this invention comprise, at least in part or fully, 2'-O-methoxy ethyl phosphorothioate RNA nucleotides or 2'-O-methyl phosphorothioate RNA nucleotides.

Oligonucleotides comprising or consisting of a sequence that is complementary to a repetitive sequence selected from the group consisting of (CAG)n, (GCG)n, (CUG)n, (CGG)n, (CCG)n, (GAA)n, (GCC)n and (CCUG)n having a length of 10 to 50, more preferably 12 to 35, most preferably 12 to 25 nucleotides, and comprising 2'-O-methoxyethyl phosphoro-

thioate RNA nucleotides, 2'-O-methyl phosphorothioate RNA nucleotides, LNA nucleotides or PMO nucleotides are most preferred for use in the invention for the diagnosis, treatment of prevention of cis-element repeat instability genetic disorders.

Accordingly, in a preferred embodiment, an oligonucleotide of the invention and used in the invention comprises or consists of a sequence that is complementary to a repetitive sequence selected from the group consisting of (CAG)n, (GCG)n, (CUG)n, (CGG)n, (GAA)n, (GCC)n and (CCUG)n, has a length of 10 to 50 nucleotides and is further characterized by:

a) comprising 2'-O-substituted RNA phosphorothioate nucleotides such as 2'-O-methyl or 2'-O-methoxy ethyl RNA phosphorothiote nucleotides, LNA nucleotides or PMO nucleotides. The nucleotides could be used in any combination and/or with DNA phosphorothioate or RNA nucleotides; and/or

b) being a single stranded oligonucleotide.

Accordingly, in another preferred embodiment, an oligonucleotide of the invention and used in the invention comprises or consists of a sequence that is complementary to a repetitive sequence selected from the group consisting of (CAG)n, (GCG)n, (CUG)n, (CGG)n, (GAA)n, (GCC)n and (CCUG)n, has a length of 10 to 50 nucleotides and is further characterized by:

c) comprising 2'-O-substituted RNA phosphorothioate nucleotides such as 2'-O-methyl or 2'-O-methoxy ethyl RNA phosphorothiote nucleotides, LNA nucleotides or PMO nucleotides. The nucleotides could be used in combination and/or with DNA phosphorothioate or RNA nucleotides; and/or

d) being a double stranded oligonucleotide.

In case, the invention relates to a double stranded oligonucleotide with two complementary strands, the antisense strand, complementary only to a repetitive sequence in a human gene transcript, this double stranded oligonucleotide is preferably not the siRNA with antisense RNA strand $(CUG)_7$ and sense RNA strand $(GCA)_7$ applied to cultured monkey fibroblast (COS-7) or human neuroblastoma (SH-SY5Y) cell lines with or without transfection with a human Huntington gene exon 1 fused to GFP and as depicted in Wanzhao Liu et al (Wanzhao Liu et al, (2003), Proc. Japan Acad, 79: 293-298). More preferably, the invention does neither relate to the double stranded oligonucleotide siRNA (with antisense strand $(CUG)_7$ and sense strand $(GCA)_7$) nor to its use for the manufacture of a medicament for the treatment or prevention of Huntington disease, even more preferably for the treatment or prevention of Huntington disease gene exon 1 containing construct.

Although use of a single oligonucleotide may be sufficient for reducing the amount of repeat expanded transcripts, such as nuclear accumulated DMPK or ZNF9 transcripts or segments thereof or sufficient reduction of accumulation of repeat expanded HD protein, it is also within the scope of the invention to combine 2, 3, 4, 5 or more oligonucleotides. The oligonucleotide comprising or consisting of a sequence that is complementary to a repetitive part of a transcript may be advantageously combined with oligonucleotides that comprise or consist of sequences that are complementary to and/or capable of hybridizing with unique sequences in a repeat containing transcript. The method of the invention and the medicaments of the invention comprising repeat specific oligonucleotides may also be combined with any other treatment or medicament for cis-element repeat instability genetic disorders.

For diagnostic purposes the oligonucleotide used in the method of the invention may be provided with a radioactive label or fluorescent label allowing detection of transcripts in samples, in cells in situ in vivo, ex vivo or in vitro. For myotonic dystrophy, labelled oligonucleotides may be used for diagnostic purposes, for visualisation of nuclear aggregates of DMPK or ZNF9 RNA transcript molecules with associated proteins. Fluorescent labels may comprise Cy3, Cy5, FITC, TRITC, Rhodamine, GFP and the like. Radioactive labels may comprise ^{3}H, ^{35}S, $^{32/33}P$, ^{125}I. Enzymes and/or immunogenic haptens such as digoxigenin, biotin and other molecular tags (HA, Myc, FLAG, VSV, lexA) may also be used. Accordingly, in a further aspect, the invention discloses an vitro or ex vivo detection and/or diagnostic method wherein a oligonucleotide as defined above is used.

The oligonucleotides for use according to the invention are suitable for direct administration to cells, tissues and/or organs in vivo of individuals affected by or at risk of developing a cis-element repeat instability disorder, and may be administered directly in vivo, ex vivo or in vitro. Alternatively, the oligonucleotides may be provided by a nucleic acid vector capable of conferring expression of the oligonucleotide in human cells, in order to allow a sustainable source of the oligonucleotides. Oligonucleotide molecules according to the invention may be provided to a cell, tissue, organ and/or subject to be treated in the form of an expression vector that is capable of conferring expression of the oligonucleotide in human cells. The vector is preferably introduced in the cell by a gene delivery vehicle. Preferred vehicles for delivery are viral vectors such as retroviral vectors, adeno-associated virus vectors (AAV), adenoviral vectors, Semliki Forest virus vectors (SFV), EBV vectors and the like. Also plasmids, artificial chromosomes, plasmids suitable for targeted homologous recombination and integration in the human genome of cells may be suitably applied for delivery of oligonucleotides. Preferred for the current invention are those vectors wherein transcription is driven from polIII promoters, and/or wherein transcripts are in the form fusions with U1 or U7 transcripts, which yield good results for delivering small transcripts.

In a preferred embodiment, a concentration of oligonucleotide, which is ranged between about 0.1 nM and about 1 μM is used. More preferably, the concentration used is ranged between about 0.3 to about 400 nM, even more preferably between about 1 to about 200 nM. If several oligonucleotides are used, this concentration may refer to the total concentration of oligonucleotides or the concentration of each oligonucleotide added. The ranges of concentration of oligonucleotide(s) as given above are preferred concentrations for in vitro or ex vivo uses. The skilled person will understand that depending on the oligonucleotide(s) used, the target cell to be treated, the gene target and its expression levels, the medium used and the transfection and incubation conditions, the concentration of oligonucleotide(s) used may further vary and may need to be optimised any further.

More preferably, the oligonucleotides to be used in the invention to prevent, treat or diagnose cis-element repeat instability disorders are synthetically produced and administered directly to cells, tissues, organs and/or patients in formulated form in pharmaceutically acceptable compositions. The delivery of the pharmaceutical compositions to the subject is preferably carried out by one or more parenteral injections, e.g. intravenous and/or subcutaneous and/or intramuscular and/or intrathecal and/or intraventricular administrations, preferably injections, at one or at multiple sites in the human body. An intrathecal or intraventricular

administration (in the cerebrospinal fluid) is preferably realized by introducing a diffusion pump into the body of a subject. Several diffusion pumps are known to the skilled person.

Pharmaceutical compositions that are to be used to target the oligonucleotide molecules comprising or consisting of a sequence that is complementary to repetitive sequences may comprise various excipients such as diluents, fillers, preservatives, solubilisers and the like, which may for instance be found in Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, Md.: Lippincott Williams & Wilkins, 2000.

Particularly preferred for the method of the invention is the use of excipients that will aid in delivery of the oligonucleotides to the cells and into the cells, in particular excipients capable of forming complexes, vesicles and/or liposomes that deliver substances and/or oligonucleotide(s) complexed or trapped in the vesicles or liposomes through a cell membrane. Many of these substances are known in the art. Suitable substances comprise polyethylenimine (PEI), ExGen 500, synthetic amphiphils (SAINT-18), Lipofectin™, DOTAP and/or viral capsid proteins that are capable of self assembly into particles that can deliver oligonucleotides to cells. Lipofectin represents an example of liposomal transfection agents. It consists of two lipid components, a cationic lipid N-[1-(2,3 dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (cp. DOTAP which is the methylsulfate salt) and a neutral lipid dioleoylphosphatidylethanolamine (DOPE). The neutral component mediates the intracellular release. Another group of delivery systems are polymeric nanoparticles. Polycations such like diethylaminoethylaminoethyl (DEAE)-dextran, which are well known as DNA transfection reagent can be combined with butylcyanoacrylate (PBCA) and hexylcyanoacrylate (PHCA) to formulate cationic nanoparticles that can deliver oligonucleotides across cell membranes into cells. In addition to these common nanoparticle materials, the cationic peptide protamine offers an alternative approach to formulate oligonucleotides as colloids. This colloidal nanoparticle system can form so called proticles, which can be prepared by a simple self-assembly process to package and mediate intracellular release of the oligonucleotides. The skilled person may select and adapt any of the above or other commercially available alternative excipients and delivery systems to package and deliver oligonucleotides for use in the current invention to deliver oligonucleotides for the treatment of cis-element repeat instability disorders in humans.

In addition, the oligonucleotide could be covalently or non-covalently linked to a targeting ligand specifically designed to facilitate the uptake in to the cell, cytoplasm and/or its nucleus. Such ligand could comprise (i) a compound (including but not limited to peptide(-like) structures) recognising cell, tissue or organ specific elements facilitating cellular uptake and/or (ii) a chemical compound able to facilitate the uptake in to cells and/or the intracellular release of an oligonucleotide from vesicles, e.g. endosomes or lysosomes. Such targeting ligand would also encompass molecules facilitating the uptake of oligonucleotides into the brain through the blood brain barrier. Therefore, in a preferred embodiment, an oligonucleotide in a medicament is provided with at least an excipient and/or a targeting ligand for delivery and/or a delivery device of the oligonucleotide to cells and/or enhancing its intracellular delivery. Accordingly, the invention also encompasses a pharmaceutically acceptable composition comprising an oligonucleotide of the invention and further comprising at least one excipient and/or a targeting ligand for delivery and/or a delivery device of the oligonucleotide to the cell and/or enhancing its intracellular delivery.

The invention also pertains to a method for the reduction of repeat containing gene transcripts in a cell comprising the administration of a single strand or double stranded oligonucleotide molecule, preferably comprising 2'-O-substituted RNA phosphorothioate nucleotides such as 2'-O-methyl or 2'-O-methoxy ethyl RNA phosphorothioate nucleotides or LNA nucleotides or PMO nucleotides, and having a length of 10 to 50 nucleotides that are complementary to the repetitive sequence only. The nucleotides could be used in combination and/or with DNA phosphorothioate nucleotides.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but combinations and/or items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

FIGURE LEGENDS

FIG. **1**: Northern blot of RNA isolated from myotubes transfected with different oligonucleotides or mock control. The myotubes were derived from immorto mouse myoblast cell lines containing a transgenic human DMPK genes with (CTG)n repeat expansion length of approximately 500 next to its normal mouse DMPK gene without (CTG) repeat. The blot shows human DMPK mRNA (top), mouse DMPK (mDMPK) mRNA (middle) and mouse GAPDH mRNA (bottom).

FIG. **2**: The human and mouse DMPK signals of FIG. **1** were quantified by phosphoimager analysis and normalized to the GAPDH signal. The results are expressed relative to the mock treatment (set to 100).

FIG. **3**: Northern blot of total RNA isolated from murine myotubes containing a mouse-human chimaeric DMPK gene in which the 3' part of the mDMPK gene was replaced by the cognate segment of the human DMPK gene including a $(CTG)_{110}$-repeat. The blot was probed for DMPK mRNA (upper panel) and mouse GAPDH mRNA (bottom). Cells were transfected with antisense oligonucleotide PS58 or control.

FIG. **4** shows the response of DM500 myotubes treated with various concentrations of oligonucleotide PS58. The expression of hDMPK was quantified via Northern blot analysis followed by phosphoimager analysis. The signal was normalised to the GAPDH signal and expressed relative to the response after mock treatment.

FIG. **5** shows the Northern blot of total RNA of DM500 myotubes transfected with 200 nM PS58 at different time points: 2 h, 4 h, 8 h and 48 h before harvesting. Mock treatment was performed 48 h before harvesting. Northern blots show human and mouse DMPK and mouse GAPDH mRNA. These were quantified by phosphoimager and the normalized DMPK signal was expressed relative to mock treatment.

FIG. **6** shows the Northern blot of total RNA of DM500 myotubes harvested 2 d, 4 d, 6 d and 8 d after transfection with 200 nM PS58 or mock control. Northern blot analysis and quantification was performed as before.

FIG. **7** shows a Northern blot of total RNA from MyoD-transformed myoblasts treated with oligonucleotide PS58 (20 and 200 nM) or mock control. The myoblasts were derived from fibroblasts obtained from a congenital myotonic dystrophy type I patient bearing a hDMPK allele with a triplet repeat expansion length of approximately 1500 and a hDMPK allele with normal length of 11 repeats. The Northern blot was hybridized with a human DMPK (hDMPK) probe and GAPDH mRNA probe. The human DMPK signals were normalized to the GAPDH signal and expressed relative to mock control.

FIG. **8** shows the RT-PCR analysis of DM500 myotubes transfected with 200 nM of oligonucleotide PS58, specific to the (CUG) repeat sequence only, oligonucleotide PS113, specific to a sequence in exon 1, or mock control. RT-PCR analysis was performed with primers specific for hDMPK mRNA and three other gene transcripts with a naturally occurring (CUG) repeat in mice: Ptbp1 mRNA with a (CUG)6, Syndecan3 mRNA with a (CUG)6 and Taxilinbeta mRNA with a (CUG)9. The intensity of the signals were normalized to the actin signal and expressed relative to mock control.

FIG. **9** shows FISH analysis of DM500 myoblasts transfected with 200 nM PS58 (B) or mock control (A). Forty eight hours after the start of the treatment, the cells were washed and fixed and subsequently hybridized with fluorescently labeled oligonucleotide Cy3-(CAG)10-Cy3. The ribonuclear foci indicative of hDMPK $(CUG)_{500}$ mRNA aggregation in the nucleus were visualized using a Bio-Rad MRC1024 confocal laser scanning microscope and LaserSharp2000 acquisition software.

FIG. **10** shows the relative cell count for the presence of ribonuclear foci in the nucleus of DM500 myoblasts transfected with PS58 or mock control from the experiment depicted in FIG. **9**.

FIG. **11** shows the RT-PCR analysis of hDMPK mRNA in muscle of DM500 mice treated with PS58 or mock control. Shortly, PS58 (2 nmol) was injected in the GPS complex of one-year-old DM500 mice and this procedure was repeated after 24 h. After 15 days, *M. plantaris* and *M. gastrocnemius* were isolated and RT-PCR was performed on total RNA for hDMPK and mouse actin. The intensity of the hDMPK signal was normalized to the actin signal and the values expressed relative to mock control.

FIG. **12** shows a Northern blot analysis of DM500 myotubes treated with different oligonucleotides (200 nM) or mock control. PS58, PS146 and PS147 carried a full 2'O-methyl phosphorothiate backbone, but differed in length, (CAG)7, (CUG)10 and (CUG)5, respectively. PS142 has a complete phosphorothiate DNA backbone with a (CAG)7 sequence. hDMPK and mDMPK signals were normalized to mouse GAPDH and expressed as percentage to mock control. Quantification is shown in the lower panel.

EXAMPLES

Example 1

Immortomyoblast cell lines were derived from DM500 or CTG110 mice using standard techniques known to the skilled person. DM500 mice were derived from mice obtained from de Gourdon group in Paris. CTG110 mice are described below and present at the group of Wieringa and Wansink in Nijmegen. Immortomyoblast cell lines DM500 or CTG110 with variable (CTG)n repeat length in the DMPK gene were grown subconfluent and maintained in a 5% CO_2 atmosphere at 33° C. on 0.1% gelatin coated dishes. Myoblast cells were grown subconfluent in DMEM supplemented with 20% FCS, 50 μg/ml gentamycin and 20 units of γ-interferon/ml. Myotube formation was induced by growing myoblast cells on Matrigel (BD Biosciences) coated dishes and placing a confluent myoblast culture at 37° C. and in DMEM supplemented with 5% horse serum and 50 μg/ml gentamycin. After five days on this low serum media contracting myotubes arose in culture and were transfected with the desired oligonucleotides. For transfection NaCl (500 mM, filter sterile), oligonucleotide and transfection reagents PEI (ExGen 500, Fermentas) were added in this specific order and directly mixed. The oligonucleotide transfection solution contained a ratio of 5 μl ExGen500 per ug oligonucleotide which is according to the instructions (ExGen 500, Fermentas). After 15 minutes of incubation at room temperature the oligonucleotide transfection solution was added to the low serum medium with the cultured myotubes and gently mixed. The final oligonucleotide concentration was 200 nM. Mock control treatment is carried out with transfection solution without an oligonucleotide. After four hours of incubation at 37° C., fresh medium was added to the culture (resulting in a dilution of approximately 2.3×) and incubation was extended overnight at 37° C. The next day the medium containing the oligonucleotide was removed and fresh low serum medium was added to the myotubes which were kept in culture at 37° C. for another day. Forty eight hours after the addition of oligonucleotide to the myotube culture (which is seven days after switching to low serum conditions to induced myotube formation), RNA was isolated with the "Total RNA mini kit" (Bio-Rad) and prepared for Northern blot and RT-PCR analysis. The Northern blot was hybridized with a radioactive human DMPK (hDMPK) probe and a mouse GAPDH probe. The probe used for DMPK is a human DMPK cDNA consisting of the DMPK open reading frame with full 3' UTR and 11 CTGs.

The human and mouse DMPK signal were quantified by phosphoimager analysis and normalized to the GAPDH signal. Primers that were used for the RT-PCR for hDMPK mRNA were situated in the 3'untranslated part with the sequence 5'-GGGGGATCACAGACCATT-3' (SEQ ID NO: 23) and 5'-TCAATGCATCCAAAACGTGGA-3' (SEQ ID NO: 24) and for murine actin the primers were as followed: Actin sense 5'-GCTAYGAGCTGCCTGACGG-3' (SEQ ID NO: 25) and Actin antisense 5'-GAGGCCAGGATG-GAGCC-3' (SEQ ID NO: 26). PCR products were run on an agarose gel and the signal was quantified using Labworks 4.0 (UVP BioImaging systems, Cambridge, United Kingdom). The intensity of each band was normalized to the intensity of the corresponding actin band and expressed relative to mock control.

Thirteen different oligonucleotides were tested (for an overview see Table 1) as described above on the immortomyoblast DM500 cell line containing transgenic human DMPK gene with (CTG)n repeat length of approximately 500 and a normal mouse DMPK gene without (CTG) repeat. FIG. **1** shows the Northern blot of the isolated RNA from the oligonucleotide transfected myotubes visualized with the hDMPK probe and a GAPDH probe for loading control. Quantification of the human DMPK (with CTG repeat) and murine DMPK (without CTG repeat) signal on the Northern blot is shown in FIG. **2**. The signal was normalized to murine GAPDH and expressed relative to mock control.

Table 2 indicates the level of hDMPK mRNA reduction that is caused by a specific oligonucleotide. The minus (−) stands for no reduction and the number of positive signs (+) stands for the relative level of hDMPK mRNA break-down. Clearly, oligonucleotide PS58, specifically targeted to the

repeat sequence, is much more potent in reducing or altering hDMPK transcripts than the other oligonucleotides complementary to unique sequences in the hDMPK transcripts.

FIG. **3** shows the effect of PS58 in murine immortomyotubes derived from CTG110 mice, a knock-in mouse containing a DMPK gene with the 3' part of the human DMPK gene including a (CTG) repeat of approximately 110. Northern blot analysis showed that the DMPK transcript containing the (CTG)110 repeat was reduced by the treatment with oligonucleotide PS58 but not after mock treatment.

Example 2 (FIG. **4**)

The DM500 immortomyoblast cell line carrying a human DMPK gene with an approximate (CTG)500 repeat expansion was cultured, prepared and transfected as described above (see example 1). In this example, the transfection was carried out with PS58 at different concentrations. Eighty four hours after start of treatment, the myotubes were harvested and Northern blot analysis was performed on isolated RNA as described above (see example 1).

FIG. **4** shows the quantification of the hDMPK mRNA signal preformed by phosphoimager analysis and normalized to the GAPDH signal at different concentrations. Under these conditions, a half maximal effect was observed at around 1 nM.

Example 3 (FIGS. **5** and **6**)

The DM500 immortomyoblast cell line carrying a human DMPK gene with an approximate (CTG)500 repeat expansion was cultured, prepared and transfected as described above (see example 1). However, in this example the transfection with 200 nM PS58 was carried out at different time points. Usually DM500 myotubes were harvested seven days after switching to low serum conditions to induce myotube formation. The standard procedure (as in example 1 and 2) was to start treatment (transfection) 48 h (two days) before harvesting. Now, treatment with PS58 was started 2 h-48 h (FIG. **5**) or 2 d-8 d (FIG. **6**) before harvesting. Northern blot analysis and quantification was performed as before.

FIG. **5** shows that expanded hDMPK mRNA in DM500 myotubes was decreased rapidly within 2 h of treatment with oligonucleotide PS58 compared to mock control treatment.

FIG. **6** shows a persistent decrease in expanded hDMPK mRNA in DM500 myotubes for at least 8 days. Please note that in the case of the 8 d experiment, cells were transfected in the myoblast stage (approximately 60% confluent, 33° C., high serum) and that they have received fresh medium on various occasions until harvesting (including a change to low serum at 37° C., two days after transfection). Example 2 and 3 are indicative of a highly efficient inhibitory intervention by an oligonucleotide directed solely to the repeat expansion. The magnitude of this effect might be influenced by the relative low levels of hDMPK expression in these model cell systems, which normally is also seen in humans.

Example 4 (FIG. **7**)

In this example, fibroblasts obtained from a human patient with congenital myotonic dystrophy type 1 (cDM1) were used. These patient cells carry one disease causing DMPK allele with a triplet repeat expansion length of 1500 and one normal DMPK allele with a repeat length of 11. The size of the (CTG)n expansion on both alleles was confirmed with PCR and Southern blotting.

The fibroblasts were grown subconfluent and maintained in a 5% CO_2 atmosphere at 37° C. on 0.1% gelatin coated dishes. Fibroblasts were grown subconfluent in DMEM supplemented with 10% FCS and 50 μg/ml gentamycin. Myotube formation was induced by growing fibroblasts cells on Matrigel (BD Biosciences) coated dishes and infecting the cells at 75% confluency with MyoD-expressing adenovirus (Ad5Fib50MyoD, Crucell, Leiden) (MOI=100) in DMEM supplemented with 2% HS and 50 μg/ml gentamycin for 2 hours. After the incubation period MyoD adenovirus was removed and DMEM supplemented with 10% FCS and 50 μg/ml gentamycin was added. The cells were maintained in this medium in a 5% CO_2 atmosphere at 37° C. until 100% confluency. At this point cells were placed in DMEM supplemented with 2% FCS and 50 μg/ml gentamycin. After five days on this low serum media cells were transfected with PS58 following the procedure according to the instructions (ExGen 500, Fermentas) and as described above. The final oligonucleotide concentration was 200 nM and 20 nM. Forty eight hours after start of the treatment (which is seven days after switching to low serum conditions), RNA was isolated with the "Total RNA mini kit" (Bio-Rad) and prepared for Northern blot. The Northern blot was hybridized with a radioactive human DMPK (hDMPK) and mouse GAPDH mRNA probe. The human DMPK signals were quantified by phosphoimager analysis and normalized to the GAPDH signal and expressed relative to mock control.

FIG. **7** shows the Northern blot analysis of the MyoD-transformed myoblasts treated with oligonucleotide PS58 (20 and 200 nM). The results demonstrate an effective complete inhibition of the disease-causing hDMPK (CUG) 1500 RNA transcript, while the smaller normal hDMPK (CUG)11 RNA transcript is only moderately affected at the two concentrations. Thus, oligonucleotides directed to the repeat region exhibit selectivity towards the larger repeat size (or disease causing expansion).

Example 5 (FIG. **8**)

In this example, the DM500 immortomyoblast cell line carrying a human DMPK gene with an approximate (CTG) 500 repeat expansion was cultured, transfected and analysed as described before in example 1. The DM500 myotubes were treated 48 h before harvesting with 200 nM of oligonucleotide PS58, specific to the (CUG) repeat sequence only, oligonucleotide PS113, specific to a sequence in exon 1, or mock control. RT-PCR analysis was performed on hDMPK mRNA expressed in this murine cell line (for primers see example 1) and on three other gene transcripts with a naturally occurring (CUG) repeat in mice, Ptbp1 with a (CUG)6, Syndecan3 with a (CUG)6 and Taxilinbeta with a (CUG)9.

The PCR primers used were for Ptbp1: 5'-TCTGTCCCTAATGTCCATGG-3' (SEQ ID NO: 27) and 5'-GCCATCTGCACAAGTGCGT-3' (SEQ ID NO: 28); for Syndecan3: 5'-GCTGTTGCTGCCACCGCT-3' (SEQ ID NO: 29) and 5'-GGCGCCTCGGGAGTGCTA-3' (SEQ ID NO: 30); and for Taxilinbeta: 5'-CTCAGCCCTGCTGCCTGT-3' (SEQ ID NO: 31) and 5'-CAGACCCATACGTGCTTATG-3' (SEQ ID NO: 32). The PCR products were run on an agarose gel and signals were quantified using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom). The intensity of each signal was normalized to the corresponding actin signal and expressed relative to mock control.

FIG. **8** shows the RT-PCR results with a maximal inhibition of hDMPK mRNA expression by PS58. The other gene transcripts carrying a naturally occurring small (CUG) repeat were not or only marginally affected by the oligonucleotide PS58, specific to the (CUG) repeat, compared to oligonucleotide PS113, which has no complementary sequence to these gene transcripts.

This example confirms the selectivity of an oligonucleotide, directed solely to the repeat region, towards the long repeat size (or disease causing expansion) compared to naturally occurring shorter repeat sizes.

Example 6 (FIG. **9** En **10**)

In this example, the DM500 immortomyoblast cell line carrying a human DMPK gene with an approximate (CTG) 500 repeat expansion was cultured and transfected with PS58 (200 nM). Here, FISH analysis was carried out on the cells. Forty eight hours after the start of the treatment, the cells were fixed with 4% formaldehyde, 5 mM $MgCl_2$ and 1×PBS for 30 minutes. Hybridization with fluorescently labeled oligonucleotide Cy3-(CAG)10-Cy3 was performed overnight at 37° C. in a humid chamber. After hybridization the material was washed and mounted in mowiol and allowed to dry overnight. Nuclear inclusions (ribonuclear foci) were visualized using a Bio-Rad MRC1024 confocal laser scanning microscope and LaserSharp2000 acquisition software. In total 50 cells were counted and scored for the presence of inclusions in the nuclei of these cells.

Literature indicates that DMPK mRNA containing a (CUG) expanded repeat accumulates and aggregates in the nucleus to form ribonuclear foci with regulatory nuclear proteins and transcription factors. Therefore, normal nuclear gene processing and cell function gets impaired.

FIG. **9** shows a mock treated cell containing ribonuclear inclusions in the nucleus, while these are no longer present in the cell nucleus after treatment with PS58. FIG. **10** shows that the percentage of nuclei containing ribonuclear foci seen under control conditions in DM500 myotubes is strongly decreased by the treatment with PS58. This result demonstrates that inhibition of hDMPK mRNA expression also inhibits the disease related triplet repeat (CUG) rich inclusions.

Example 7 (FIG. **11**)

Here, the effect of PS58 was evaluated in vivo in DM500 mice containing hDMPK with a (CTG)n expansion of approximately 500 triplets. The DM500 mice were derived by somatic expansion from the DM300 mouse (e.g. see Gomes-Pereira M et al (2007) PLoS Genet. 2007 3(4): e52). A (CTG) triplet repeat expansion of approximately 500 was confirmed by southern blot and PCR analysis.

In short, PS58 was mixed with transfection agent ExGen 500 (Fermentas) according to the accompanying instructions for in vivo use. PS58 (2 nmol, in the transfection solution with Exgen 500) was injected (40 μl) in the GPS complex of one-year-old DM500 mice and this procedure was repeated after 24 h. As a control, DM500 mice were treated similarly with the transfection solution without PS58. After 15 days, the mice were sacrificed, muscles were isolated and total RNA was isolated from the tissues (using Trizol, Invitrogen). RT-PCR analysis was performed to detect hDMPK mRNA in the muscle similar as described above. The intensity of each band was performed using the Labworks 4.0 program (UVP BioImaging systems, Cambridge, United Kingdom) and normalized to the intensity of the corresponding actin band. Primer location is indicated in the figure.

FIG. **11** shows that in vivo treatment of DM500 mice with PS58 strongly reduced the presence of hDMPK mRNA containing a (CUG)n repeat expansion compared to mock treatment in the *M. plantaris* and *M. gastrocnemius*.

Example 8 (FIG. **12**)

In this example, different oligonucleotides (in length and backbone chemistry) but all with a sequence directed solely to the (CTG)n repeat expansion were compared. DM500 myotubes were cultured, transfected and analysed as described above in example 1. Northern blots were quantified by phosphoimager analysis and DMPK signals were normalized to GAPDH.

Here, the DM500 myotubes were treated with the following oligonucleotides (200 nM), all with a complete phosphorothioate backbone (see Table 3).

FIG. **12** shows that treatment of the DM500 myotubes results in a complete reduction of (CUG)n expanded hDMPK mRNA for all oligonucleotides tested. Under the present conditions, the maximal effect obtainable is independent of oligonucleotide length, backbone modification or potential mechanism of inhibition by the employed single stranded oligonucleotides.

Example 9

Fibroblasts (GM 00305) from a male patient with Huntington's Disease were obtained from Coriell Cell Repository (Camden, N.J., US) and cultured according to the accompanying instructions and standard techniques known to the skilled person in the art. Huntington patients carry one healthy and one disease-causing allele of the Huntington gene resulting in the expression of both mRNAs with respectively a normal number and an expanded number of (CAG) repeats, respectively.

The fibroblasts were transfected with a 21-mer 2'O-methyl phosphorothioate RNA antisense oligonucleotide PS57 with a (CUG)7 sequence, complementary to the (CAG) triplet repeat in Huntington mRNA. Transfection occurred at 100 or 200 nM in the presence of PEI as indicated by the manufacturer. Twenty four hours after transfection the cells were harvested and total RNA was isolated and analysed by RT-PCR. The Huntington transcript was determined using primers in downstream exon 64 (5' GAAAG TCAGT CCGGG TAGAA CTTC 3' (SEQ ID NO: 33) and 5' CAGAT ACCCG CTCCA TAGCA A 3' (SEQ ID NO: 34)). This method detects both types of Huntington mRNAs, the normal and mutant transcript with the additional (CAG) expansion. GAPDH mRNA (housekeeping gene) was also determined. The signals were quantified and the total amount of Huntington mRNA was normalised to the amount of GAPDH mRNA in the same sample. The results are expressed relative to a control treated (without oligonucleotide) sample from fibroblasts (which was to 100%). In the samples from fibroblasts transfected with either 100 or 200 nM of PS57, significantly lower levels of total Huntington mRNA levels were observed of approximately 53% and 66% compared to the levels in control-treated cells, respectively.

Thus, PS57, an oligonucleotide directed only to the (CAG) repeat, induces a decrease in Huntington mRNA levels and these results are consistent with a selective inhibition of mutant over normal Huntington mRNA.

TABLE 1

Overview oligonucleotides tested

Oligo name	Modification	Sequence	Position
PS40	2'OMe RNA phosphorothioate/FAM	GAGGGGCGUCCAGGGAUCCG	intron 14-exon 15
PS41	2'OMe RNA phosphorothioate	GCGUCCAGGGAUCCGGACCG	intron 14-exon 15
PS42	2'OMe RNA phosphorothioate	CAGGGAUCCGGACCGGAUAG	intron 14-exon 15
PS56	DNA	CAGCAGCAGCAGCAGCAGCAG	repeat in exon 15
PS58	2'OMe RNA phosphorothioate/FAM	CAGCAGCAGCAGCAGCAGCAG	repeat in exon 15
PS59	2'OMe RNA phosphorothioate	UGAGUUGGCCGGCGUGGGCC	ESE exon 15
PS60	2'OMe RNA phosphorothioate	UUCUAGGGUUCAGGGAGCGCGG	ESE exon 15
PS61	2'OMe RNA phosphorothioate	ACUGGAGCUGGGCGGAGACCC	ESE exon 15
PS62	2'OMe RNA phosphorothioate	CUCCCCGGCCGCUAGGGGGC	ESE exon 15
PS113	DNA phosphothioroate	GAGCCGCCTCAGCCGCACCTC	Exon 1
PS114	DNA phosphothioroate	GAAGTCGGCCACGTACTTGTC	Exon 1
PS115	DNA phosphothioroate	GGAGTCGAAGACAGTTCTAGG	Exon 15
PS116	DNA phosphothioroate	GGTACACAGGACTGGAGCTGG	Exon 15

TABLE 2

Reduction of hDMPK mRNA after oligo transfection:

Oligo	Reduction hDMPK mRNA	SEQ ID No. 's
PS40	+	1
PS41	–	2
PS42	–	3
PS59	–	4
PS60	–	5
PS61	+/–	6
PS62	–	7
PS58	++++	8
PS56	–	9
PS113	–	10
PS114	–	11
PS115	+/–	12
PS116	+	13

(–) indicates no reduction, (+) indicates level of reduction in hDMPK mRNA.

TABLE 3

Oligonucleotides used in example 9

#	Length	(CAG)n	Substitution ribose	RNAse H breakdown possible
PS58	21-mer	n = 7	2'O-Methyl	No
PS146	30-mer	n = 10	2'O-Methyl	No
PS147	15-mer	n = 5	2'O-Methyl	No
PS142	21-mer	n = 7	Deoxyribose (DNA)	Yes

* all oligonucleotides full length phosphorothioate and substitution

SEQUENCE LISTING

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<223> OTHER INFORMATION: chemically synthesized oligonucleotide PS41

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cugcugcugc ugcugcugcu g 21

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<400> SEQUENCE: 36

gccgccgccg cc 12

The invention claimed is:

1. A method of treating a spino-cerebellar ataxia (SCA), spinal and bulbar muscular atrophy (SBMA), or dentatorubral-pallidoluysian atrophy (DRPLA) in a subject in need thereof, comprising administering to the subject an oligonucleotide comprising or consisting of a sequence that is complementary only to a polyglutamine (CAG)n repetitive nucleotide unit in a gene transcript.

2. The method of claim **1**, wherein the SCA is SCA type 1, 2, 3, 6, 7 or 17.

3. The method of claim **1**, wherein said oligonucleotide has a length of 10 to 50 nucleotides.

4. The method of claim **3**, wherein said oligonucleotide has a length of 12 to 30 nucleotides.

5. The method of claim **4**, wherein said oligonucleotide is a single-stranded oligonucleotide.

6. The method of claim **1**, wherein said oligonucleotide comprises or consists of RNA nucleotides, DNA nucleotides, 2'-O substituted RNA nucleotides, locked nucleic acid (LNA) nucleotides, peptide nucleic acid (PNA) nucleotides, morpholinophosphorodiamidates, ethylene-bridged nucleic acid (ENA) nucleotides or mixtures thereof, with or without a phosphorothioate-containing backbone.

7. The method of claim **6**, wherein the oligonucleotide comprises 2'-O substituted RNA phosphorothioate nucleotides.

8. The method of claim **7**, wherein said 2'-O-substituted RNA phosphorothioate nucleotide is a 2'-O-methyl or 2'-O-methoxy ethyl RNA phosphorothioate nucleotide.

9. The method of claim **1**, wherein said oligonucleotide is provided in an expression vector.

10. The method of claim **9**, wherein said expression vector is a viral vector.

11. The method of claim **1**, wherein said oligonucleotide is provided with an excipient and/or a targeting ligand for delivery of the oligonucleotide to cells and/or for enhancing intracellular delivery of the oligonucleotide.

12. The method of claim **1**, wherein said oligonucleotide is comprised in a pharmaceutically acceptable composition.

13. The method of claim **12**, wherein said pharmaceutical composition further comprises at least one excipient and/or targeting ligand for delivery of the oligonucleotide to the cell and/or for enhancing intracellular delivery of the oligonucleotide.

14. The method of claim **1**, wherein said oligonucleotide preferentially hybridizes to a disease-associated or disease-causing transcript and leaves the function of a normal transcript relatively unaffected.

15. The method of claim **1**, wherein said oligonucleotide prevents the accumulation and/or translation of repeat expanded transcripts in cells.

16. The method of claim **15**, wherein said repeat expanded transcript is a (CAG)n repeat in an ATXN1, ATXN2, ATXN3, SCA7, CACNA1A, AR, SCA17 or DRPLA gene transcript in a cell.

17. The method of claim **1**, wherein said oligonucleotide interferes with gene expression or one or more other precursor RNA or messenger-RNA dependent cellular processes.

18. The method of claim **17**, wherein said messenger-RNA-dependent cellular process is RNA splicing or exon skipping.

19. The method of claim **1**, wherein said administration is carried out by one or more parenteral injections at one or multiple sites in the human body.

20. The method of claim **19**, wherein said parenteral injection is an intravenous, a subcutaneous, an intramuscular, an intrathecal, or an intraventricular injection.

21. A method of treating a spino-cerebellar ataxia (SCA) in a subject in need thereof, comprising administering to the subject the oligonucleotide of claim **1**.

22. A method of treating spinal and bulbar muscular atrophy (SBMA) in a subject in need thereof, comprising administering to the subject the oligonucleotide of claim **1**.

23. A method of treating dentatorubral-pallidoluysian atrophy (DRPLA) in a subject in need thereof, comprising administering to the subject the oligonucleotide of claim **1**.

24. A method of treating a spino-cerebellar ataxia (SCA), spinal and bulbar muscular atrophy (SBMA), or dentatorubral-pallidoluysian atrophy (DRPLA) in a subject in need thereof, comprising administering to the subject an oligonucleotide comprising or consisting of a sequence that is complementary only to a polyglutamine (CAG)n repetitive nucleotide unit in a gene transcript, wherein the oligonucleotide comprises at least one modification and 10 to 50 nucleotides that are complementary to CAGCAGCAGCAG (SEQ ID NO: 18).

25. A method of treating a spino-cerebellar ataxia (SCA), spinal and bulbar muscular atrophy (SBMA), or dentatorubral-pallidoluysian atrophy (DRPLA) in a subject in need thereof, comprising administering to the subject an oligonucleotide comprising or consisting of a sequence that is complementary only to a polyglutamine (CAG)n repetitive nucleotide unit in a gene transcript, wherein the oligonucle-

otide comprises a sequence selected from the group consisting of: SEQ ID NOs: 5 (cug cug cug cug cug cug cug) and 20 (cug cug cug cug).

* * * * *